Final Report

on

Project NR 151-151 ONR Contract Nonr-762(00)

ANTIGENIC AND CULTURAL PROPERTIES OF NOCARDIA

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INTRODUCTION

This project was undertaken because it was felt that the existing state of knowledge regarding the antigenic properties and relationships among the described Nocardia species, as well as the possible relationships among Nocardia and Mycobacteria, was inadequate. It was anticipated that the study to be undertaken would lead to a better understanding of these relationships and thus to improved knowledge of the biology of the Nocardia. It was also hoped that certain of the results might be of value in solving the sometimes difficult problem of differential etiologic diagnosis in human infection with acid-fast microbes. As indicated in the original request for support, the not uncommon presence of Nocardia in human tissues or products may create confusion in diagnosis and prognosis, unless the true nature of the infecting organisms is recognized. While some species are probably merely contaminants or secondary invaders as are saprophytic mycobacteria (e.g., 1), other species are undoubtedly capable of causing serious or even fatal disease so that their proper identification is important (e.g., 2). It seemed definitely worthwhile, therefore, to investigate the possibility that suitable serologic tests might be developed which would permit of more rapid and accurate methods for diagnosis.

As a preliminary to the serologic study it was considered desirable to carry out a comparative study of the morphologic and cultural behavior of the respective microorganisms. While recent taxonomic classifications of Nocardia include enumeration of these properties for numerous species (e.g., 3, 4) such summaries are of necessity based at

least in part on the original literature, made up of articles some of which describe only a single or few species. Certain of the descriptions are, moreover, incomplete or inadequate by current standards, while some of the original type strains are no longer available. For the present study, we endeavored to secure from various sources in this country and Great Britain what we considered to be a reasonably representative collection of previously identified strains of Nocardia, including both saprophytic and known pathogenic strains. We also selected for comparative study certain saprophytic species of Mycobacteria which were available in our laboratory. The collection of cultures was then examined for cultural behavior employing methods and media which we believed might yield data of differential value, particularly since for purposes of uniformity the various strains were inoculated into samples of a given batch of medium at approximately the same time.

In the serologic studies which followed the cultural work, an effort was made to propagate all of the cultures on a uniform semi-synthetic fluid medium, free of large molecular weight impurities, so as to avoid inadverdent production of antibodies to constituents of the medium. The medium selected was one which would support the abundant growth of most Nocardia and saprophytic Mycobacteria; it would also be satisfactory for pathogenic mycobacterial species in case we should wish to study the latter in subsequent phases of the work. A good deal of effort was expended in developing convenient methods for production of suspensions of the microbes sufficiently stable to be used in agglutina-

tion tests. Various technics for performing the agglutination test were also examined and it was concluded that a macroscopic slide test had definite advantages over a macroscopic tube test or a microscopic slide test.

It was hoped on the basis of past experience with other antigens that chickens would prove satisfactory in the production of high-titered specific antisera for Nocardia and indeed the preliminary data obtained with such antisera seemed quite encouraging. It was only after considerable work had been done that we found many chicken sera to be capable of agglutinating Nocardia suspensions even prior to experimental immunization of the animals, so it was decided to abandon further attempts in this direction and to use rabbits instead. The individual rabbits were selected on the basis of tests of their sera, which were collected on pre-immunization bleeding and set up against the antigen which the animals were expected to receive in subsequent inoculations.

Rabbits with little or no evidence of pre-immunization antibodies were found to be satisfactory for production of antisera, although the dilution titers obtained did not in general have high numerical values and many animals required multiple series of injections to obtain a stable peak titer. Numerous animals also died during the course of immunization presumably as a result of the endotoxic properties of the antigenic preparations.

Since it was decided to test all antisera simultaneously with a given antigen and because of the unavoidable delay in securing these reagents, they were stored for prolonged periods. In the case of the

antisera which were stored at 4-5°C after addition of glycerin (33 per cent by volume), successive tests carried out at intervals gave some evidence that the agglutinating capacity had deteriorated. Other technical factors, described below, also gave some difficulty but were believed overcome by the time the project was terminated. In spite of the foregoing, it is felt that the serologic data presented are of considerable interest and we feel that they shed much light on procedures to be used in future studies which might be expected to be of even greater value.

Note: Since the earlier literature concerning serologic tests, especially agglutination tests, with actinomycetes has been reviewed by Slack, Ludwig, Bird and Canby (6), no attempt has been made to analyse it in this report. It may be mentioned, however, that many of the investigators used unidentified cultures of aerobic actinomycetes or strains designated by names which are difficult to correlate with those in current use. In some instances work was performed with only a few species and with single strains of a given species. Various workers have noted serologic cross-reactions among aerobic actinomycetes and saprophytic or avirulent mycobacteria, as well as low-titered reactions between certain Nocardia and M. tuberculosis. The relationships were more readily demonstrated by complement-fixation than by agglutination tests. It seems fair to say in summary that the examination of the problem of serologic relationship among Nocardia and Mycobacteria is far from complete.

I. CULTURAL STUDIES

A. Microbial Collection Examined

The cultural characteristics of the following species and strains were investigated:

Culture Designation			Obtained from	O.S.N.+
Nocardia				
N. asteroides	ATCC*	3308	U. S. Army Med. Res. and Grad. School	316
" "	"	9504	" " " " " " " "	312
" "	"	9970	" " " " " " " "	314
" "	strain	312	Dr. N. F. Conant, Duke Univ.	304
" "	"	314	" " " " " "	302
" "	"	-	" " " " " "	308
" "	"	Henrici	Univ. of Minnesota	310
" "	"	Wojik 23	U.S.P.H.S. Hospital, New Orleans	306
N. blackwellii	ATCC	6846	Am. Type Culture Collection	315
N. caviae	"	6848	U. S. Army Med. Res. and Grad. School	315
N. corallina	"	999	Dr. R. L. Starkey, Rutgers Univ.	371
N. cuniculi	"	6864	Am. Type Culture Collection	325
N. erythropolis	"	4277	U. S. Army Med. Res. and Grad. School	367
N. farcinica	"	3318	Dr. E. H. Ludwig, West Virginia Univ.	332
N. globerula	"	9356	U. S. Army Med. Res. and Grad. School	317
N. intracellularis	-		Dr. N. F. Conant, Duke Univ.	330
N. leishmanii	ATCC	6855	Am. Type Culture Collection	336

+ O.S.N. = Our serial number

* ATCC = American Type Culture Collection

Culture Designation	Obtained from	O.S.N.
N. madurae ATCC 6245	Dr. E. H. Ludwig	335
N. madurae -	Dr. N. F. Conant, Duke Univ.	333
N. mexicana Waksman 18	" " " " " "	326
" " " 24	" " " " " "	322
" " strain 2178	" " " " " "	324
N. minima ATCC 8674	U. S. Army Med. Res. and Grad. School	309
N. opaca " 4276	" " " " " " " "	319
N. paraffinâe strain 3410	Dr. R. L. Starkey	362
N. paraguayensis strain 285	Dr. G. Ochoa, Mexico City	307
N. pelletieri " 293	" " " " "	305
N. polychromogenes ATCC 3409	Am. Type Culture Collection	370
N. rangoonensis " 6860	" " " "	323

Mycobacteria

M. butyricum	Tulane Dept. of Microb.	264
M. leprae, strain Duval	" " " "	266
M. phlei, strain 125	" " " "	260
M. smegmatis	" " " "	270
M. stercoris	" " " "	262

B. Methods of Study

Unless otherwise specified below, all cultures were maintained at room temperature; this varied from 24° to 32°C during the period of the cultural studies.

1. Growth on Solid Media

Each strain was cultured on slants of the following media: Pai's egg; 4 per cent glycerin heart-infusion agar pH 6.8; Sabouraud's agar; Czapek's agar; 3 per cent sheep blood heart-infusion agar; carrot agar; brain heart infusion agar; potato glucose agar; 0.2 per cent starch in glucose neopeptone agar. The nature of the growth was observed after 15 and 25 days.

Characteristics which were recorded included: production of aerial mycelium, pigmentation of the surface growth, ability to discolor the medium (diffusible pigment), gross appearance and consistency of the growth.

2. Slide Culture

In preparing slide cultures, sterile equipment and aseptic precautions were employed. Two capillary glass rods were placed in parallel across the center of a slide, the distance separating the rods being slightly less than the width of a coverglass. In the center of the space between the rods a very small fragment of culture mass was placed and overspread with a coverglass which rested on the rods. Melted glycerin agar at about 50-55°C was then pipetted from one side to fill the space between coverglass and slide up to the level of the inoculum, so that the latter was imbedded just at its surface on solidification of the medium. The

space between the slide and the edge of the coverglass was sealed on three sides with a mixture containing equal parts of melted paraffin and vaseline, the side left open being the one opposite to that at which the agar had been introduced. The slide mount was next placed in a petri dish containing a small wad of moist cotton and the development of the culture was observed at intervals of 2-3 days over a period of 2 weeks. Thereafter the coverslip was removed; the adhering growth was fixed with heat and stained with crystal violet, then attached to a slide with Permount. The preparations were observed for the production of branched mycelium and for the degree to which the latter fragmented.

3. Acid-fast Staining Properties

Smears were made from cultures on glycerin agar and in brom-thymol-blue (BTB) milk medium, after 10 days of cultivation. The staining solution and method employed were those of Kinyoun, thus eliminating the heating of the preparations; decolorization was attempted by immersion of the smears for 10 seconds in either 1 per cent H_2SO_4 solution or in 3 per cent HCl solution. As long as some red microbial elements were subsequently seen on microscopic examination of the smear, the culture was considered to be acid-fast although a distinction was made between weakly and strongly acid-fast varieties.

4. Growth at 42° and 52°C

Triplicate sets of cultures were made on glycerin agar, one set being subsequently incubated at 42°C and a second at 52°C while a third set was kept at room temperature to check on the viability of the inoculum. Cultures were observed at intervals over a 15-day period.

5. Paraffin utilization

Czapek's fluid medium (without added sugar and containing ammonium nitrate as the sole source of nitrogen) was inoculated by emulsifying a small particle of growth (from a glycerin agar slant) against the wall of the tube at the level of the upper air-fluid interface. A glass rod coated with sterile paraffin was then inserted into the tube so that a length of at least one centimeter of the paraffin-coated portion remained above the surface of the medium. The cultures were observed for evidence of growth at intervals of 15 and 30 days respectively.

6. Starch Hydrolysis

Each strain was inoculated by streaking down the center of the surface of 2 long thin slants of neopeptone glucose agar containing 0.2 per cent soluble starch. One set of cultures was tested after 10 days, the second set after 25 days, by pouring 7-8 drops of Gram's iodine solution over the surface of the slant and reading after a 5-minute interval of standing. With diastatic strains, the medium showed no trace of the color which would be seen if starch were still present; with non-diastatic strains a purplish to blue-black color developed almost at once over the entire surface of the medium.

7. Nitrate Reduction

Two sets of cultures were prepared on nitrate agar, one being tested at the end of 10 days and the other after 30 days. The sulfanilic acid and dimethyl-alpha-naphthylamine reagents were each added in 0.5 ml quantities and the tubes were shaken to assure wetting of the surface of the slant. The test was read after allowing the tubes to stand for 5 minutes. Where only a faint trace of pink developed in the surface of

the medium with no color in the reagent, the reaction was considered doubtful. Positive reactions were graded +, ++ and +++ based on the increasing intensity of the color.

8. Gelatin Liquefaction

In cool weather the nutrient gelatin medium was first liquefied by placing the tubes in warm water prior to inoculating; in hot weather this was unnecessary. The inoculum, derived from a stock glycerin-agar slant culture, was thoroughly distributed throughout the medium which was put up in Wassermann tubes. At intervals of 14, 30 and 40 days respectively the tubes were placed in the cold room (at 4-5°C) for 2 hours and observed there for persistent liquefaction. It was noted that when liquefaction occurred it was always evident by the 30th day so that incubation for a longer period was not necessary.

9. Action on Brom-thymol-blue Milk

Brom-thymol-blue was chosen as the indicator, partly because its range of color change with varying pH extends on both the acid and alkaline side of neutrality and partly because it was noted in preliminary tests that litmus was reduced to a colorless form during growth of the Nocardia whereas BTB was not so affected. The inoculated medium was observed after intervals of 10, 15, 30 and 50 days respectively; evidence of pH change and other enzymatic action were sought. Clearing of the turbidity of the milk was considered to indicate peptonization. Later experiments have shown that this effect can be much more readily demonstrated by growing the organisms on agar slants containing milk, instead of in fluid milk.

Table I.
Summary of Certain Cultural Properties of Strains of *Nocardia* and *Mycobacteria*

Our Serial No.	Strain Studied	Gross Culture: Aerial Mycelium Present on: Pal's Egg	Gly. Agar	Sab. Agar	Czapek Agar	Carrot Agar	Potato Glucose Agar	Starch Agar	Gross Culture: Color of Growth: Yellow Orange or Red	White Cream Buff Tan or Brown	Gross Culture: Diffusible Pigment in Medium: Pal's Egg	Gly. Agar	Sab. Agar	Starch Agar	Slide Culture: Aerial Hyphae: Branched	Fragmented	Slide Culture: Submerged Growth Present	Acid Fastness in Culture Medium: Gly. Agar After Attempted Decolorization with H_2SO_4	Gly. Agar After Attempted Decolorization with HCl	Milk After Attempted Decolorization with H_2SO_4	Milk After Attempted Decolorization with HCl	Growth at 42	Growth at 52	Paraffin Utilized	Starch Hydrolyzed	Nitrite from Nitrate	Gelatin Liquefied	Milk Fermentation of: pH Change	Peptonization	Glucose	Lactose	Galactose	Mannite	Raffinose	Xylose	Rhamnose	Hemolysis
316	N. asteroides	+	+	+	-		-	+	+	-	-	-	-	-	+		+	+	-	+	-	+	+	+	-	+	-	Alk	-	-	-	-	-	-	-	-	-
312	" "	+	+	+	+		+	+	+	-	-	-	-	-	+		+	+	+	++	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
314	" "	+	+	+	+	+	+	+	+	-	-	-	-	-	+		+	+	++	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
304	" "	-	-	-	-	-	-	+	+	-	-	-	-	-	+		+	+++	-	++	-	+	-	+	-	+	-	Alk	-	-	-	-	-	-	-	-	-
302	" "	+	+	+	+	+	+	+	+	-	+	+	+	+	+		+	+++	+	+++	+	+	-	+	-	-	-	Alk	-	-	-	-	-	-	-	-	-
308	" "	+	-	+	+	-	+	+	+	-	-	-	-	+	+		+	++	-	+++	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
310	" "	+	+	+	+	+	-	+	+	-	+	+	+	+	+		+	+	+	++	+	+	-	+	-	-	-	Alk	-	-	-	-	-	-	-	-	-
306	" "	-	+	+	+	+	+	+	+	-	+	+	-	+	+		+	++	+	+++	+	+	-	+	-	+	-	Alk	-	-	-	-	-	-	-	-	-
313	N. blackwellii	-	-	+	-	-	-	+	+	-	-	-	-	-	+		+	++	-	+	-	+	-	+	-	-	-	Alk	-	-	-	-	-	-	-	-	-
315	N. caviae	+	+	+	-	-	-	+	-	+	+	+	-	-	+		+	-	-	-	-	+	-	+	+	-	+	Alk	-	-	-	+	-	+	+	-	+
371	N. coralline	-	-	-	-	-	-	-	+	-	-	+	-	-	-		+	-	-	+	-	+	-	-	-	++	-	Alk	-	-	-	-	-	-	-	-	-
325	N. cuniculi	-	+	-	-	-	-	+	+	-	-	+	-	-	+		+	+	-	+	-	+	-	+	-	-	-	Alk	-	-	-	-	-	-	-	-	-
367	N. erythropolis	-	-	-	-	-	-	-	+	-	-	-	-	-	-		+	-	-	+	-	+	-	+	-	+	-	Alk	-	-	-	-	-	-	-	-	-
332	N. farcinica	+		-	-	+	-	+	-	+	-	+	-	+			+	+	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	+
317	N. globerula	-	-	-	-	-	-	-	+	-	-	-	+	-	-		+	-	-	-	-	-	-	+	-	++	-	Alk	-	-	-	-	-	-	-	-	-
330	N. intracellularis	-	-	-	-	-	-	-	-	+	-	-	-	-	-		+	-	++++	++	++++	+++	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
336	N. leishmanii	-	-	-	-	+	+	+	-	+	+	+	-	-	+		+	-	-	-	-	-	-	-	+	+	-	+	Alk	+	-	-	-	-	-	-	-	+
335	N. madurae	+	+	+	+	+	+	+	-	+	+	+	+	-	+		-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-	-	-	+
333	N. "	-	-	-	-	+	+	+	-	+	-	-	-	-	+		+	-	-	-	-	-	-	-	+	-	+++	+	-	-	-	-	-	-	-	-	+

Table I. (continued)
Summary of Certain Cultural Properties of Strains of <u>Nocardia</u> and <u>Mycobacteria</u>

Our Serial No.	Strain Studied	Characteristics of Gross Culture: Aerial Mycelium Present on Pai's Egg	Gly. Agar	Sab. Agar	Czapek Agar	Carrot Agar	Potato Glucose Agar	Starch Agar	Color of Growth: Yellow Orange or Red	White Cream Buff Tan or Brown	Diffusible Pigment in Medium: Pai's Egg	Gly. Agar	Sab. Agar	Starch Agar	Slide Culture: Aerial Hyphae Branched	Fragmented	Submerged Growth Present	Acid Fastness in Culture Medium After Attempted Decolorization with: Gly. Agar H_2SO_4	Gly. Agar HCl	Milk H_2SO_4	Milk HCl	Growth at 42	52	Paraffin Utilized	Starch Hydrolyzed	Nitrite from Nitrate	Gelatin Liquefied	pH change	Milk Peptonization	Fermentation of Glucose	Lactose	Galactose	Mannite	Raffinose	Xylose	Rhamnose	Hemolysis
326	N. mexicana	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	++	-	++	-	-	-	+	-	+	+	Alk	+	-	-	-	-	-	-	-	-
322	N. "	-	+	-	+	+	+	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	+	-	+	+	AC	+	-	-		-	-	-	-	-
324	N. "	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	-	+	-	-	-	+	-	++	+	Alk	+								
309	N. minima	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	+++	++	-	-	+	-	-	-	-	-	-	-	+	-	+		+	-
319	N. opaca	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	++	+	+	-	+	-	++	-	Alk	-	-	-	-	-	-	-	-	-
362	N. paraffinae	-	-	-	-	+	-	+	+	-	-	+	-	-	+	+	+	+	-	++	+	-	-	+	-	++	-	-	-		-		-		-	-	-
307	N. paragueyensis	-	-	-	-	-	-	+	-	+	-	-	+	+				-	-	-	-	-	-	-	-	+++	+			+		+	+	+		-	+
305	N. pelletieri	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-		-	-	-	-	+	-	-	-	+++	-			-		-	-				-
370	N. polychromogenes	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	++	+	-	-	+	-	+	-	Alk	-		-	-	-		-	-	-
323	N. rangoonensis	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	-	-	-	-	+	-	+	-	-	+	AC	+	-	+	+	-	+	-	-	-
264	M. butyricum	-	-	-	-	-	-	-	+	-	-	-	-		-	+	-	++++	++	++++	+++	+	+	+	-	-	-	Alk	-	-	-	-	-	-	-	-	-
266	M. leprae	-	-	-	-	-	-	-	+	-	-	-	-		-	+	-	++++	++	++++	+++	+	+	+	-	+	-	AC	-	-	-	-	-	-	-	-	-
260	M. phlei	-	-	-	-	-	-	-	+	-	-	-	-		-	+	-	++++	-	++++	+++	-	-	+	-	+	-	Alk	-	-	-	-	-	-	-	-	-
264	M. smegmatis	-	-	-	-	-	-	-	+	-	-	-	-		-	+	-	++++	-	++++	+++	+	+	+	-	-	-	Alk	-	-	-	-	-	-	-	-	-
262	M. stercoris	-	-	-	-	-	-	-	+	+	-	-	-		-	+	-	++++	-	++++	+++	+	+	+	-	-	+	Alk	-	-	-	-	-	-	-	-	-

10. Carbohydrate Fermentation

The following carbohydrates were added in 1 per cent concentration to serum broth (pH 7.0) containing BTB as indicator; glucose, maltose, lactose, sucrose, galactose, arabinose, mannite, raffinose, rhamnose, xylose and inulin. Sets of tubes containing the above media were inoculated for each strain and observed for acid production at intervals up to 30 days.

11. Hemolysis

Plates of 3 per cent sheep blood heart-infusion agar were inoculated for each strain and examined at intervals over a period of 3 weeks for evidence of zones of hemolysis.

C. Experimental Results

The data on the cultural tests mentioned above are summarized in Table I. A few remarks on some of the findings seem worthy of mention.

1. Characteristics of Growth on Solid Media

a) Aerial mycelium - When the various cultures were examined for production of aerial mycelium (the development of a white fuzzy, chalky or powdery surface on the growth), all the strains of N. asteroides were found to do so to some extent but this varied with the media on which they were grown. The 3 strains of N. mexicana also produced aerial mycelium on most media. Other strains of Nocardia were variable; some (strains with a soft pasty or yeast-like type of growth) did not produce an aerial mycelium on any of the media, as was true also for the 5 strains of Mycobacteria.

While growth on potato glucose agar and Sabouraud's agar was usually likely to show evidence of aerial mycelium, the most consistently positive

results were secured with starch agar.

The observation that several Nocardia strains did not form aerial mycelium on any of the media which were used might be explained either on the basis of the loss of this ability by the microorganisms on prolonged maintenance in the laboratory or on a deficiency in the substrate. Other workers, however, have made similar observations and it would appear that Nocardia strains or species vary inherently in their ability to produce a well-developed gross aerial mycelium (3). In any case, the characteristic is too variable to be acceptable as a prime criterion for differentiating Nocardia from Mycobacteria.

Mycobacteria

b) Pigment production - This is a characteristic of Nocardia which (as for other microbes) varies with temperature; the pigment produced in the growth at 42°C was noted to be much paler than that produced at room temperature. The intensity of the pigment also varied with the medium, although the basic color remained fairly constant. All strains of N. asteroides produced growth exhibiting some shade of yellow or orange pigment; other Nocardia also produced similar, and occasionally red, pigments. Still other strains of Nocardia produced only cream, buff to brown pigments and never yellow, orange or red. The production of the buff to brown pigment appeared to be correlated with lack of acid-fastness and failure of the mycelium to fragment. Because of the wide range of colors it was difficult to draw a sharp line of demarcation between color groups.

Discoloration of the medium appeared to be a variable characteristic, depending not only on the particular strain of a given Nocardia species

but also to some extent on the substrate used for cultivation. The Mycobacteria did not discolor the media on which they were grown.

It was concluded that pigment production is probably of limited value in identification but should be correlated with other activities.

c) Consistency of growth - This varied from a soft, pasty, bacteria-like growth (e.g., N. corallina, N. globerula) to a very firm, leathery, almost rigid turf (some strains of N. asteroides and others). In between these two extremes there was almost every possible intermediate condition so that it was extremely difficult to make any separation into groups on the basis of growth consistency alone. The consistency varied with the medium used. It was felt that the use of growth consistency as a basis of separating groups should be avoided.

2. Growth in Slide Culture

When the Nocardia were grown in slide culture a recognizable true mycelium was usually observed to develop and to creep out onto both glass surfaces ahead of the fragmented portion. The only exception among all the Nocardia strains studied was N. intracellularis in which fragmentation was so complete, even in young cultures, that no mycelium could be recognized. Among the remaining Nocardia strains fragmentation varied, some persisting unfragmented regardless of the age of the culture; with other strains, although fragmentation was usually complete in old cultures one could see (out near the tip of the growth) bacillary elements still in the position originally occupied by the unfragmented filament and its branches. Submerged growth was commonly seen.

The Mycobacteria which were studied in slide cultures showed only a packed mass of bacillary elements; there was no submerged growth.

3. Acid-fastness

All strains of N. asteroides studied showed some degree of acid-fastness although considerable variation existed among them and they were in general, more acid-fast after being grown in milk than on glycerin agar. Among the other species studied many were not at all acid-fast. Those forms that showed fragmentation of the mycelium were acid-fast, with the exception of one strain. All strains that did not fragment were also non-acid-fast. Since acid-fastness appears to vary with the type of medium used and the method of staining, it should probably not be used as a primary characteristic in identification except when carried out with adequate consideration of these factors.

4. Growth at 42° and 52°C

In nearly all cases where growth occurred at the higher temperatures it was quite definite and easily observed, in some instances being even more profuse than at room temperature. It was of interest that while all 8 strains of N. asteroides grew well at 42°C, none of the 5 N. mexicana strains were capable of doing so. The other Nocardia differed similarly in their ability to grow at 42°C. Only 2 of the total collection of 29 Nocardia strains could grow (poorly) at 52° whereas all 5 saprophytic Mycobacterium species grew well even at the higher temperature.

5. Paraffin Utilization

When proliferation occurred it developed only on the surface of the paraffin at and just below the surface of the fluid medium, forming around the rod a band 2-4 mm. wide. Growth did not occur in the depths of the medium and no pellicle was produced.

Most of the strains examined, both Nocardia and Mycobacteria, could utilize paraffin. There was no complete correlation between this capability and other activities. The 4 Nocardia strains which were unable to utilize the hydrocarbon substrate were completely or nearly non-acid-fast, but other non-acid-fast species did grow well on this material.

6. Starch Hydrolysis

Apart from four non-acid-fast Nocardia strains which proved to be diastatic, the great majority of Nocardia and the 5 mycobacterial species did not give evidence of such activity.

7. Nitrate Reduction

This property appeared to be variable in occurrence and degree and without apparent relationship to other characteristics of the strains.

8. Gelatin Liquefaction

Eight of the 10 non-acid-fast strains of Nocardia were capable of liquefying gelatin while most of the acid-fast Nocardia and Mycobacteria did not do so. There was however no absolute correlation between these characteristics.

9. Behavior in BTB Milk

Growth of most of the Nocardia strains in this medium resulted in either an alkaline reaction or no change in the indicator; only 2 strains produced acid. The 3 strains of N. mexicana and 3 other (non-acid-fast) Nocardia strains peptonized the milk. None of the Mycobacterium cultures gave evidence of peptonization; one produced an acid reaction.

10. Carbohydrate Fermentation

The Mycobacteria and most of the Nocardia showed little or no fermentative activity when grown in the presence of various carbohydrates commonly used in bacteriologic work. Maltose, sucrose, arabinose and inulin were not fermented by any of the strains tested. Four of the 29 Nocardia strains effected color changes in the media suggesting that they might have produced acid from certain of the substrates and these strains were all non-acid-fast. The carbohydrates apparently fermented varied from one strain to another and did not seem to fit with any obvious pattern. The possibility is not excluded, however, that the four strains might have reduced the BTB indicator and concomitantly have produced a yellow to brown soluble pigment. This point requires further checking.

11. Hemolysis

A few species of Nocardia, all of them non-acid-fast, produced zones of hemolysis on sheep blood agar. All of the acid-fast species, as well as a few non-acid-fast varieties, were non-hemolytic under the experimental conditions.

II. SEROLOGIC STUDIES

A. Preparation of Reagents

1. Propagation of Cultures on "Standard" Medium

In an attempt to cultivate all the strains on a uniform semi-synthetic fluid medium which itself would be free of antigenic components, a modified Long-Seibert medium was prepared and distributed in quantities of 150 ml. in 1-liter Florence flasks which were plugged with ordinary non-

absorbent cotton and sterilized by autoclaving. The composition of the medium was as follows:

Casamino acids (Difco)	10 gm.
Asparagin	0.5 gm.
Potassium acid phosphate	3.0 gm.
Sodium carbonate (anhydrous)	3.0 gm.
Sodium chloride	2.0 gm.
Magnesium sulfate	1.0 gm.
Ferric ammonium citrate	0.05 gm.
Glycerin	40.0 ml.
Distilled water	1000 ml.

For each strain, six flasks were inoculated by tilting to expose the bottom on which was emulsified (with a spatula) a bit of 10-day growth from glycerin agar slants. The flask was then slowly returned to the upright position, allowing the inoculum to float on the surface of the fluid. By the end of 30 days at room temperature an abundant wrinkled waxy growth was present on the surface of the clear medium. Contamination was infrequent, but if present, it was detected by the following checks: a) observation of the medium for cloudiness, or discrete surface colonies (of adventitious fungi); b) streaking of an aliquot from each flask on a plate of blood agar and incubation of the latter at 37°C for 2 days, followed by 1 week at room temperature; c) examination of plates of Littman's oxgall agar which had been streaked with an aliquot from each flask and incubated 1 week at room temperature. The identity of the waxy microbial pellicle on the fluid medium was further checked by transferring to glycerin agar slants some of the typical

growth recovered on the blood agar plate, then comparing the gross cultural characteristics of the resultant culture with those of the strain employed as the original inoculum.

For each strain, the uncontaminated fluid cultures were shaken and pooled in a 4-liter flask. Phenol was added to 0.5 per cent final concentration and the mixture stored at 4°C for 5 days, after which the contents of the flask were filtered through sterile filter paper. Of the filtrate, all but 100 ml (which was stored in a sterile bottle at 4°C) was discarded.

Four strains (N. asteroides OSN 304, N. blackwellii OSN 313, N. madurae OSN 333 and N. opaca OSN 319) grew very slowly on the fluid medium employed and produced such scant growth that it was decided to propagate them on solid substrate in order to obtain sufficient material for antigen preparation. Good growth was obtained on the modified Long-Seibert medium solidified by the addition of 2 per cent agar. The resultant microbial mass was harvested (by simply scraping it off the surface of the slant) and thereafter processed in the same manner as the growth harvested from the fluid medium following the filtration procedure mentioned above.

2. Preparation of Antigen Suspensions

Unstandardized antigen suspensions were prepared for each of the following strains of organisms.

O.S.N.	Species
302	Nocardia asteroides
304	" "
306	" "
308	" "
310	" "
312	" "
314	" "
316	" "
322	" mexicana
324	" "
326	" "
309	" minima
313	" blackwellii
315	" caviae
317	" globerula
319	" opaca
323	" rangoonensis
325	" cuniculi
330	" intracellularis
332	" farcinica
333	" madurae
336	" leishmanii
362	" paraffinae
370	" polychromogenes
371	" corallina
260	Mycobacterium phlei
270	" smegmatis

O.S.N.	Cultural Designation	Obtained from
318	Nocardia asteroides	undetermined source
320	" " 14A1	Dr. A. Kligman, Univ. of Penna.
342	" " A402	USPHS Communicable Disease Center
346	" " A343	" " " "
348	" " A144	" " " "
350	" " A267	" " " "
352	" " 80-Henrici 4139	" " " "
354	" " 2001	Dr. S. Salvin, USPHS, Rocky Mt. Lab.
356	" " -	Dept. Vet. Path., Ohio State Univ. (isolated from dog)
334	" salmonicolor	Dr. D. Erikson, England
101	Streptomyces griseus ATCC3326A	Am. Type Culture Collection
102	" lavendulae ATCC8664	" " " "
103	" venezuelae ATCC10595	" " " "

The strains listed above the double line are the same strains on which the cultural studies described in part I were carried out. The strains listed below the double line were received much later and no cultural studies had been done on this group. This latter group of organisms was also received subsequent to the completion of the immunization procedures so that no antisera were prepared against them. The antigens prepared from these strains were however standardized and later checked against available antisera.

The harvested microbial mass derived from either fluid or solid medium was now treated to obtain a dense (unstandardized antigen) suspension as follows. The solid growth was transferred to a sterile mortar and was manually ground with the aid of a pestle and an equal volume sterile washed Hi-Flo Supercel which was added. To the resultant homogeneous paste was added enough phenolized saline diluent (0.15 M sodium chloride solution buffered at pH 7.2 - 7.4 with 0.025 M potassium phosphates and containing 0.2 per cent phenol plus 0.1 per cent bile salts (Difco) to make a semi-fluid but easily flowing suspension. The latter was passed twice through a Wood-Werkman cone mill* and thereafter diluted sixfold with the phenolized saline diluent. The diluted suspension was next spun 10 minutes at 500 rpm on the horizontal head of a centrifuge, in order to sediment the abrasive as well as gross particles of the antigen. The deposit was discarded, the supernate (unstandardized antigen suspension) was transferred to sterile stoppered bottles and was stored at 4-5°C until needed.

* Kindly made available by Dr. A. G. C. White of the Tulane Dept. of Biochemistry.

It should be pointed out that in the early stages of the work attempts were made to prepare the suspension simply by grinding with saline in a mortar but without the addition of abrasive. This proved unsatisfactory, in terms of the lack of uniformity and stability of the microbial particles, so the use of the Wood-Werkman cone mill was tried. Even with this apparatus it was found difficult to prepare satisfactory homogeneous suspensions until the use of Hi-Flo Supercel (Johns-Manville Co.) was adopted. With the aid of the abrasive, stable suspensions were secured and all of the antigens used below were prepared in the manner described above. In the latter stages of our work, it was found that satisfactory suspensions could be prepared by simply grinding the microbial mass in a mortar with Supercel and eliminating the stage of secondary grinding in the cone mill.

For the production of antisera, unstandardized antigen suspensions were injected. After the immunization procedure was completed and before being used for agglutination tests, all antigens were matched against a selected optical density standard and adjusted to give a suspension equivalent in turbidity to a suspension of E. coli containing 4.05×10^9 organisms per ml.

3. Preparation of Antisera

Prior to immunization of all animals, 40 ml. of blood was withdrawn by cardiac puncture and the serum was stored for later use as a control reagent. All blood taken, whether pre-or post-immunization, was defibrinated with glass beads and the serum then separated by centrifugation. As a preservative c.p. glycerin (pH 7.2, adjusted by the addition of sodium

hydroxide) was added, one part by volume to two parts of serum. The glycerinated serum was stored in screw-cap glass bottles at 4-5°C. A preliminary determination of the titer of each antiserum was made immediatly after it was secured, according to the following procedure. A series of doubling dilutions of both pre- and post-immunization serum were made in buffered saline, ranging from 1:10 to 1:160. Two parallel rows of squares having been ruled off with crayon on a glass plate, one drop of each dilution was placed in squares arranged so a given dilution of pre-immunization (control) serum was adjacent to the similar dilution of hyper-immune serum. To each drop of serum was added one drop of unstandardized undiluted homologous antigen. The plate was placed on a Yankee rotator (Clay-Adams) and rotated in the horizontal plane to 130 R.P.M. for 5 minutes. The mixtures were then examined for evidence of easily recognized gross agglutination, holding the plate over a fluorescent light. Agglutination requiring inspection with a hand lens for recognition was not considered as positive in the readings.

It was first decided to try chickens as a source of antibody to the Nocardia. Ten chickens were used, each being immunized against a different strain of N. asteroides. Each chicken was given 2 ml. antigen intravenously and 2 ml. intraperitoneally, at 5-day intervals for a total of 4 injections. Following a week's rest, each chicken was bled for titer check and the series of injections was then repeated until each chicken had received a total of three series of inoculations. After the first titer check it became evident that chickens would be unsatisfactory for use with the Nocardia. In all but one of the sera obtained, the pre-immunization specimen agglutinated the antigen to the

same titer as the post-immunization samples. In some cases the agglutination in the pre-immunization serum was stronger than in the post-immunization sample. It was therefore decided to use rabbits for the production of antibody.

For each strain or species used as antigen, 2 rabbits were employed. Because of limited facilities in the animal house, immunization could not be carried out against more than 8 to 10 strains at any one time. This had certain drawbacks, since by the time the last group of immunizations was completed the sera obtained from the first group were over a year old. Variations in the quantity of inoculum and route of inoculations were tried in the different groups of rabbits used. On each occasion of injection the vaccine was given either intravenously only, or both intravenously and intraperitoneally; the interval between successive injections in a given series varied from 4 to 7 days. The quantity administered by either route varied in different series from 3 to 10 ml.; in a given series, vaccine was injected on 3 to 10 occasions. The animals were bled 7 days after the last injection in each series. Each animal received 1 to 3 series of injections, depending on the promptness with which antibodies were produced.

The results may be summarized as follows:

a) When a given antigen was administered to a pair of rabbits, the response observed was generally similar in rate and degree. In many instances the serum of both animals showed similar titers or titers varying by not more than 1 or 2 doubling dilutions, when bleedings were taken at the same time. In other cases, the serum titer in one rabbit of a pair was significantly lower after the first series of injections but when the

Table II.
Summary of Agglutination Titers in Tests Employing Buffered Saline pH 7.2 as Diluent

ANTISERA from Rabbit	Strain	vs. O.S.N.	ANTIGENS O.S.N.	316	312	314	304	302	308	310	306	318	320	313	315	371	325	332	317	330	336	333	326	322	324	309	319	362	370	323	260	270	101	102	103
				N. asteroides	"	"	"	"	"	"	"	"	"	N. blackwelltii	N. caviae	N. corallina	N. cuniculi	N. farcinica	N. globerula	N. intracellularis	N. leishmanii	N. madurae	N. mexicana	"	"	N. minima	N. opaca	N. paraffinae	N. polychromogenes	N. rangoonensis	M. phlei	M. smegmatis	S. griseus	S. lavendulae	S. venezuelae
202	N. asteroides		312	160	160	-*		x+				160	160	-	x	10	40		80	-	80	-	160			-	10								
237	" "		304	80	20	10	80	x	80	20	20	40	40	20	x	80	40	20	160	-	80	40	x			80	20	10	80	10	10	80	-	-
208	" "		302	-	-	-	-	x	-	-	x	-	-	-	x	-	-	-	-	-	x	x	-	-	80	-	-	-	-	-	-	-	-	-
246	" "		308	-	40	-	x	x	x	-	x	40	80	-	x	-	-	-	x	-	x	x	-	-	x	-	-	-	x	-	x	-	x	-	-
251	" "		306	20	20	20	40	x	160	-	40	20	80	80	x	160	80	40	160	40	160	x	10	x	x	40	20	40	80	20	x	40	160	40	80
348	N. globerula		317	-	-	-	-	x	-	-	-	-	-	-	x	-	-	-	160	-	x	-	-	-	-	10	-	-	-	-	-	-	-	-	-
243	N. intracellularis		330	-	-	-	-	x	-	-	-	-	-	-	x	-	-	-	-	600	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-
347	N. mexicana		322	20	40	20	80	x	160	40	40	40	160	80	x	80	40	-	160	-	80	x	80	400	400	-	-	-	80	-	-	-	80	80	-
233	M. phlei		260	20	10	20	10	x	20	10	-	20	40	10	x	80	20	20	80	10	80	x	-	20	20	10	10	20	40	10	400	20	10	-	-

* - signifies a titer of less than 1:10

+x signifies agglutination in the pre-immunization (control) specimen of a given rabbit's serum.

hyper-immunization was continued similar level[illegible] were attained after the second or third series.

b) Certain antigens stimulated an antibody resp[illegible]nse which was demonstrable in both rabbits after the first series of injec[illegible]ions, while with a few strains no agglutinins were detected even after [illegible] third series. For many strains two series of injections were requi[illegible]d for production of suitable titers, but there was little difference [illegible]between the titers attained at the conclusion of the second and thir[illegible] series.

c) The titers attained, as measured by the proced[illegible]e employed, were not high; they ranged from 1:10 to 1:320, with many sera [illegible]showing titers of 1:40 to 1:160.

d) No consistent difference was noted in the results o[illegible]tained which could be attributed to the quantity of vaccine admini[illegible]red, number of injections, route of injections or interval between [illegible]tions.

B. Procedure for Performing Agglutinati[illegible]sts

As in the preliminary determinations of homologou[illegible] titer (see above), the comparative tests were carried out on glass plates [illegible]8" x 10") ruled off with wax pencil into six longitudinal rows of five [illegible]squares each. Pre-immunization (control) serum dilutions were added to t[illegible]e squares in one of each adjacent pair of rows, dilutions of post-immu[illegible]nization serum from the same animal were added to the squares in the seco[illegible]nd row of the pair; thus three different antigens could be tested at the [illegible]same time on a given plate. Each square received 0.03 ml of a serum dilu[illegible]tion and nearby 0.03 ml of an antigen, each measured from a 20 gauge-needle [illegible]attached to a tuberculin syringe (0.25 ml capacity) graduated in div[illegible]isions of 0.01 ml. When

all the various reagents had been dispensed on the squares of the plate, the antigen and serum dilutions were mixed with a wire and the plate was set on the horizontal rotator run at 130 R.P.M. for 5 minutes. The plate was then removed, held above a fluorescent light and examined for gross agglutination. Our methods thus differed from those used by Ludwig, Slack and their collaborators (5, 6) who within the past several years have also been engaged in studies on serologic relationships among actinomycetes.

All comparative tests were carried out with standardized antigen suspensions (see above). For the sera a series of 5 successive doubling dilutions was made in a given diluent, starting with 1:10 dilution. In those instances where marked agglutination occurred in the highest such serum dilution (1:160), the test was repeated with dilutions of serum as follows: 1:100, 1:200, 1:400, 1:600.

C. Results of Agglutination Tests

The first series of comparative tests was begun on Feb. 10, 1953 and was carried out with sera diluted in 0.01 M phosphate-buffered saline pH 7.2 - 7.4. The results are summarized in Table II but certain points seem worthy of brief mention here. Considering first the behavior of the respective antisera:

a) Of the 5 antisera prepared with as many different strains of N. asteroides, one (#208) seemed to be essentially devoid of agglutinating antibody and this antiserum had resulted from injections of N. asteroides O.S.N. 302 which appeared to be agglutinated non-specifically by all the sera against which it was tested. It is possible that, as with certain strains

of other microbes, N. asteroides O.S.N. 302 may have been antigenically degraded and lacking in type-specific surface components which would otherwise have rendered it stable in the presence of normal serum. Two anti-N. asteroides sera (202 and 246) seemed to be relatively specific, in that the post-immunization specimens agglutinated some but not all other N. asteroides strains tested and they did not agglutinate many other species of Nocardia nor the Mycobacteria and Streptomyces strains tested. Two other anti-N. asteroides sera (237 and 251) appeared to be less specific and more broadly reactive in their behavior when set up against various Nocardia, Mycobacteria and Streptomyces antigens.

b) The anti-N. globerula and anti-N. intracellularis sera appeared to be relatively specific and potent against their respective homologous organisms but showed little or no evidence of increased titer of the post-immunization specimens when tested against heterologous species.

c) The anti-N. mexicana serum appeared to react with nearly all N. asteroides strains tested; it also agglutinated some, but not all, other Nocardia species. It agglutinated 2 of 3 Streptomyces species, but not the 2 Mycobacterium species tested.

d) The anti-M. phlei serum showed low-titered cross-reactions with various Nocardia strains but a significantly higher titer with the homologous antigen. It was noteworthy that this antiserum showed only low-titered reaction versus M. smegmatis and insignificant reactivity with Streptomyces antigens.

Examination of the behavior of the various antigens led to the following tentative conclusions:

a) N. asteroides O.S.N. 302 and N. caviae O.S.N. 315 appeared to be spontaneously agglutinable.

b) The other 4 N. asteroides strains appeared to be antigenically heterogeneous, since some of them were not agglutinated by antisera which proved capable of agglutinating other strains. Antigens from N. asteroides strains O.S.N. 304, 306, and 308 were agglutinated by pre-immunization serum specimens from one or more animals. None of the N. asteroides strains were agglutinated by the anti-N. globerula or anti-N. intracellularis sera, while they were all agglutinated by the anti-N. mexicana serum and to a lesser degree also by the anti-M. phlei serum.

c) N. blackwellii and N. farcinica showed a similar pattern of behavior, differing chiefly in the reactivity with anti-N. mexicana serum exhibited by the former.

d) N. intracellularis appeared to be only slightly affected by antisera prepared against heterologous species.

e) N. minima, N. opaca, N. paraffinae, N. polychromogenes, N. rangoonensis showed only moderate degrees of cross reactivity with heterologous antisera.

f) N. leishmanii showed cross reactions with certain anti-N. asteroides antisera as well as with anti-N. mexicana and anti-M. phlei serum.

g) The N. madurae antigen was agglutinated by pre-immunization bleedings of 3 anti-N. asteroides sera as well as the control specimens of the anti-M. phlei and anti-N. mexicana sera. It was not agglutinated by single antiserum vs. N. asteroides, N. globerula or N. intracellularis.

h) The N. mexicana antigens showed some cross reactions with anti-N. asteroides sera but produced higher titers with antiserum to the homologous species.

i) The M. phlei antigen appeared to be little affected by other than homologous antiserum; the anti-M. phlei serum did not however agglutinate M. smegmatis to a corresponding degree.

j) The Streptomyces antigens were only agglutinated by one or two anti-N. asteroides antisera and by anti-N. mexicana serum.

During the course of performing the tests in the first series, it was noted that a considerable degree of agglutination occurred in pre-immunization (control) sera, particularly when N. asteroides strains O.S.N. 308 and 312 were used. In an effort to eliminate such behavior, these strains were tested against sera diluted with 4 per cent bovine albumin solution in buffered saline pH 6.8 and it was observed that the use of this diluent eliminated most of the agglutination produced by the control sera without any appreciable change in the titer of the post-immunization sera. It was therefore decided to carry out a second series of tests using the bovine albumin solution in buffered saline as a diluent.

In May, 1953 the second series of tests was undertaken, in which the only modification in procedure was the substitution of the albumin solution for the original diluent. Results are shown in Table III. For some of the strains sera were available from both rabbits of a pair which had received injections of a given antigen. In certain instances the reactivity of both such sera showed a fairly good degree of correspondence; when this was the case the results for the two antisera have been recorded on a single line, but where the reactivity differed significantly, the behavior of each serum is recorded separately. It should be stated also that no evidence of a reaction with any antigen was obtained in trials

Table III.

Summary of Agglutination Titers in Tests Employing as Serum Diluent a 4% Solution of Bovine Albumin in Buffered Saline pH 6.8

Rabbits from	Strain	OSN	O.S.N.	ANTIGENS	302	304	306	308	310	312	314	316	318	320	342	346	348	350	352	354	356	322	324	326	309	313	317	319	323	325	330	332	333	337	334	336	362	370	371	260	270	101	102	103
					N. asteroides	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	N. mexicana	"	"	"	N. minima	N. blackwellii	N. globerula	N. opaca	N. rangoonensis	N. cuniculi	N. intracellularis	N. farcinica	N. madurae	"	N. salmonicolor	N. leishmanii	N. paraffinae	N. polychromogenes	N. corallina	L. phlei	L. smegmatis	S. griseus	S. lavendulae	S. venezuelae
208; 281	N. asteroides			302	10	-	-	-	-	-	-	-	-	-																															
					20																																							
237	"	"		304	10	40	-	40	20	20	20	20	10	40																															
251; 255	"	"		306	-	-	80	-	10	20	-	-	-	-																															
246	"	"		308	-	20	-	-	-	80	-	-	80	40																															
254	"	"		310	-	-	-	-	80	-	-	-	-	-																															
230; 202	"	"		312	-	-	-	-	-	80	-	-	-	-																															
										160																																		
278	"	"		316	10	40	10	40	20	40	-	80	20	20		10	20			160																								
213	"	"		318	10	10	-	10	-	-	10	10	40	=	10	-	40	10	-	80																								
232; 323	"	"		320	-	-	10	-	-	10	-	-	-	10																														
347; 305	N. mexicana			322	-	-	-	-	80	10	-	10	40	-	-	-	40	-	x	-	-	200	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
					20	40				20						40							80	10		40		80															
315	"	"		324	-	-	-	-	-	-	-	-	-	-	-	-	20	-	-	-	-	-	80	10	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
299	"	"		324	20	40	40	-	80	20	40	20	40	-	20	10	-	10	20	80	80	20	80	-	-	-	80	-	10	80	-	-	-	-	-	10	20	80	-	-	-	-	-
288	"	"		326	-	-	-	-	-	-	10	20	-	20	-	20	-	-	80	40	20	-	-	10	-	10	20	-	-	10	-	10	10	-	10	40	10							
223; 285	N. blackwellii			313	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
437	N. globerula			317	-	-	-	-	-	-	-	10	-	-	10	-	10	-	-		80		-	-	-	-	400	10	-	-	-	-	-	-	10	-	-	80	-	-	-	-	-	-
438	"	"		317	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-		-	-	-	200	-	-	-	-	-	-	-	-	-	-	40	-	-	-	-	-	-
238	N. rangoonensis			323	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-		-	-	-	-	-	-	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

with antisera prepared against the following organisms N. asteroides O.S.N. 314, N. minima O.S.N. 306, N. opaca O.S.N. 318, N. cuniculi O.S.N. 325. These tests are therefore excluded from the table, likewise the tests with antigen of N. cavias O.S.N. 315 which appeared to be non-specifically agglutinated by all sera.

In surveying the results shown in Table III, the following points appear to be worthy of mention:

a) The anti-N. asteroides sera varied in their ability to react with individual strains of this organism. The antisera to N. asteroides strains O.S.N. 310 and 312, for example, appeared to be relatively specific for the homologous organisms and showed no cross-reactions with other strains of this species. The antisera to strains O.S.N. 304, 306, 316, and 318 appeared more broadly reactive, while the low-titers obtained with antisera to strains O.S.N. 302 and 320 suggested that the suspensions used for inoculation of rabbits had been poorly antigenic. Where a given antiserum reacted with several strains of N. asteroides, however, differences in titer were noted suggesting antigenic heterogeneity among the strains of this organism

b) The anti-N. asteroides sera showed little evidence of cross-reaction with N. farcinica, N. intracellularis, N. leishmanii, N. madurae, N. mexicana, N. minima, N. rangoonensis; or the mycobacterial and streptomycetal antigens tested. Cross-reactions in two or more anti-N. asteroides were obtained with N. blackwellii, N. corallina, N. cuniculi, N. globerula, N. paraffinae and N. polychromogenes.

c) One of the anti-N. mexicana sera appeared to be relatively specific;

the other antisera appeared more broadly reactive, although the highest titers for the anti-N. mexicana O.S.N. 322 sera were obtained with the homologous organism. The data suggested that strains O.S.N. 322 and 324 might be antigenically distinguishable. Paradoxically, the reactions of the respective homologous antigens with anti-N. mexicana O.S.N. 326 serum and with anti-N. blackwellii O.S.N. 313 serum appeared to be of lesser degree than was obtained with heterologous organisms.

d) The antisera of N. globerula O.S.N. 317 appeared to be relatively specific, yielding highest titers with the homologous antigen; the significant cross-reactions appeared to be limited to N. corallina and one or more strains of N. asteroides.

e) The anti-N. rangoonensis O.S.N. 323 serum appeared to be quite specific for its homologous antigen.

f) Antigens of the following strains were not agglutinated to a significant titer by any of the heterologous antisera tested. N. lactamdurans O.S.N. 332, N. intracellularis O.S.N. 330, N. madurae O.S.N. 337, N. minima O.S.N. 309, M. smegmatis O.S.N. 270, S. griseus O.S.N. 101, S. venezuelae O.S.N. 103.

After many strains had been tested in the system employing bovine albumin solution, it appeared that the agglutinability of some antigens was being adversely affected in the presence of this diluent. For example, anti-N. madurae O.S.N. 333 serum diluted in bovine albumin solution did not agglutinate the homologous antigen even at a titer of 1:10, whereas the preliminary check of serum titer performed shortly after the time of bleeding had given a value of 1:160. N. madurae O.S.N. 333 antigen was therefore tested against aliquots of the homologous antiserum diluted 1:20 and 1:40 with 4.0, 2.0, 1.0, 0.2 and 0.1 per cent solutions of

bovine albumin respectively. No agglutination was seen in antiserum diluted with saline containing bovine albumin at concentrations of 1.0 per cent or greater. In 0.2 per cent albumin solution good agglutination was obtained and the reaction was much more marked in the 0.1 per cent solution. The antigen was then re-checked against antiserum diluted 1:10 through 1:160 solely in buffered saline; definite agglutination was seen even in the highest dilution, as had been the case originally. It was concluded that the presence of excessive bovine albumin inhibited agglutination of some of the antigens and further tests using this reagent were discontinued.

On October 15, 1953 a third series of tests was begun, using 0.01 molar phosphate buffered saline as serum diluent. The results in general were similar to those obtained in the earlier series although some differences were observed. It was noted, however, that many of the antisera which had exhibited satisfactory titers versus the homologous organisms when tested shortly after bleeding or in the first series now failed to show agglutination even in the 1:10 dilution. It was concluded tentatively that some of the antibodies might be labile on storing or that glycerin might not be a satisfactory preservative. The latter conclusion was reinforced by the observation that many of the antisera had developed cloudiness and at least three had become contaminated. At this point the project was terminated.

III. SUMMARY

1. Cultural Studies

A collection of previously identified Nocardia strains was secured

...ural behavior together with that of

and tested to compare their ... behaved

a few saprophytic mycobacterial strains. Most of the ...

according to the descriptions given for them in the literature, although some divergences were observed.

The property of producing grossly visible aerial mycelium(on the surface of solid media) was found to be relatively easily and regularly demonstrable with some Nocardia strains while with others it was lacking or demonstrable only on certain media.

The production of branched aerial hyphae visible on microscopic examination of slide cultures was demonstrable for the majority of Nocardia strains, but was lacking for certain Nocardia and all of the saprophytic Mycobacteria tested. Only a few strains of Nocardia (notably N. caviae, N. leishmanii, N. madurae, N. pelletieri, N. rangoonensis) failed to show marked fragmentation of the hyphae. Most strains of Nocardia exhibited submerged growth of mycelium ... culture, while the Mycobacteria tested did not.

Pigment production and the consistency of ...owth were variable characteristics.

Acid-fastness was variable and when demonstrable was much less pronounced for Nocardia than for Mycobacteria. In degree it was dependent largely on the medium used for cultivation of the strain examined and also on the method of attempted decolorization.

The majority of Nocardia and the saprophytic Mycobacteria were capable of growing at 42°C, but only a few of the former grew at 52°C.

Most strains of Nocardia were capable of utilizing paraffin for growth, as were the saprophytic Mycobacteria. N. corallina, N. farcinica,

N. paraguayensis and N. pelletieri appeared unable to utilize paraffin.

Diastatic activity and nitrate reduction were variable among the strains examined.

Gelatin liquefaction was observed for certain of the strains isolated from man or animals (some N. asteroides strains, also N. caviae, N. leishmanii, N. madurae, N. mexicana, N. paraguayensis, N. farcinica). Some, but not all, of the strains capable of liquefying gelatin, N. rangoonensis, was also capable of peptonizing milk, but the majority of [illegible] and the Mycobacteria did not acidify or peptonize milk.

Of these strains which exhibited proteolytic activity, all but N. asteroides, N. mexicana and N. rangoonensis showed hemolytic activity against sheep erythrocytes.

2. Serologic Studies

Methods were developed for performing macroscopic slide agglutination tests using suspensions of the respective microbes and antisera obtained by repeatedly injecting rabbits with antigens prepared from cultures grown on a semi-synthetic medium. In one series of tests, buffered saline was used as diluent for the antiserum; in a second series, 4 per cent solution of bovine albumin in buffered saline was used as the diluent. It appeared from subsequent work that the bovine albumin diluent, while reducing the tendency of Nocardia suspensions to agglutinate in pre-immunization sera, also interfered to some extent with the reactions in post-immunization specimens. Nevertheless, the following tentative conclusions seem warranted from the combined data.

a) Some strains of N. asteroides evoked the formation of antibodies cross-reacting with other strains of this species as well as with other Nocardia.

b) Some strains of N. asteroides appeared to evoke the production of relatively specific antisera which showed little tendency to react with heterologous antigens.

c) Antisera produced by injections of N. globerula, N. intracellularis and N. rangoonensis appeared to be relatively specific for the homologous organisms.

d) Certain N. mexicana strains employed evoked in some rabbits antibodies which reacted broadly with a wide variety of other Nocardia including N. asteroides, while other antisera were more narrowly specific. There was limited evidence of a degree of antigenic specificity among the N. mexicana strains.

e) There was relatively little cross-reactivity exhibited by the various anti-Nocardia sera when tested against M. phlei, M. smegmatis or three Streptomyces strains.

f) The anti-M. phlei serum showed a relatively high homologous titer but also gave cross-reactions in low titer with many strains of Nocardia.

IV. CONCLUSION

The application of serological testing to a collection of identified Nocardia species and strains, as well as to a few strains of Streptomyces and Mycobacteria, has revealed further evidence of the value of such procedures for the investigation of relationships among the poorly-studied aerobic actinomycetes.

Considerable profit can be expected from further work along the lines which have thus far been explored, using methods similar to (or modified from) those developed in this investigation.

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